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Aux/IAA Proteins Repress Expression of Reporter Genes Containing Natural and Highly Active Synthetic Auxin Response Elements

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A highly active synthetic auxin response element (AuxRE), referred to as DR5, was created by performing site-directed mutations in a natural composite AuxRE found in the soybean *GH3* promoter. DR5 consisted of tandem direct repeats of 11 bp that included the auxin-responsive TGTCTC element. The DR5 AuxRE showed greater auxin responsiveness than a natural composite AuxRE and the *GH3* promoter when assayed by transient expression in carrot protoplasts or in stably transformed Arabidopsis seedlings, and it provides a useful reporter gene for studying auxin-responsive transcription in wild-type plants and mutants. An auxin response transcription factor, ARF1, bound with specificity to the DR5 AuxRE in vitro and interacted with Aux/IAA proteins in a yeast two-hybrid system. Cotransfection experiments with natural and synthetic AuxRE reporter genes and effector genes encoding Aux/IAA proteins showed that overexpression of Aux/IAA proteins in carrot protoplasts resulted in specific repression of TGTCTC AuxRE reporter gene expression.

INTRODUCTION

The plant hormone auxin plays important roles in plant growth and development, at least in part, by regulating gene expression. A number of early or primary response genes that are induced by auxin have been identified and characterized (reviewed in Abel and Theologis, 1996). One of these genes, *GH3* from soybean, contains an auxin-responsive promoter with at least three auxin response elements (AuxREs) that can function independently of one another (Liu et al., 1994). Two of the AuxREs in the *GH3* promoter have been shown to function as composite elements, consisting of a TGTCTC element and an adjacent or overlapping coupling element (Ulmasov et al., 1995). The coupling element confers constitutive expression (i.e., identical activities in the presence or absence of auxin) to a minimal promoter–β-glucuronidase (*GUS*) reporter gene when the TGTCTC element is mutated or deleted. With composite AuxREs, the TGTCTC element causes repression of *GUS* reporter gene expression in the absence of auxin and activation of expression in the presence of auxin. Gain-of-function experiments with minimal promoter–*GUS* reporter genes have shown that a single copy of a composite AuxRE is sufficient to confer auxin responsiveness to reporter genes but that a single copy of the TGTCTC element by itself has no activity (Ulmasov et al., 1995).

The smallest composite AuxRE identified to date is the D1-4 element from the *GH3* promoter. The D1-4 AuxRE is 11 bp (i.e., 5′-CCTCGTGTCTC-3′) and contains a coupling element that overlaps with the TGTCTC element (Ulmasov et al., 1995). Here, we report on site-directed mutations in the 5′ end of the D1-4 AuxRE that resulted in an exceptionally strong AuxRE (referred to as DR5). Cotransfection of effector plasmids that express Aux/IAA proteins and *GUS* reporter genes with minimal promoters fused to natural or DR5 AuxREs indicated that Aux/IAA proteins specifically repress expression from TGTCTC AuxREs.

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RESULTS

Creation of a Highly Active Synthetic AuxRE

TGTCTC AuxREs were fused upstream of a minimal −46 cauliflower mosaic virus (CaMV) 35S promoter–*GUS* reporter gene and tested for activity and auxin inducibility by using transient assays with carrot protoplasts. Figure 1A shows that a D1-4(8×) construct, containing eight tandem copies of the 11-bp natural D1-4 AuxRE from the *GH3* promoter (Ulmasov et al., 1995), was several-fold more active than a minimal CaMV 35S promoter–*GUS* reporter gene and was induced approximately fivefold by 25 μM 1-naphthalene acetic acid (1-NAA). Site-directed mutations were made in the D1-4 AuxRE, and these mutations were tested as eight tandem copy constructs. The D1-5(8×) construct contained a 2-bp substitution within the middle of the TGTCTC element

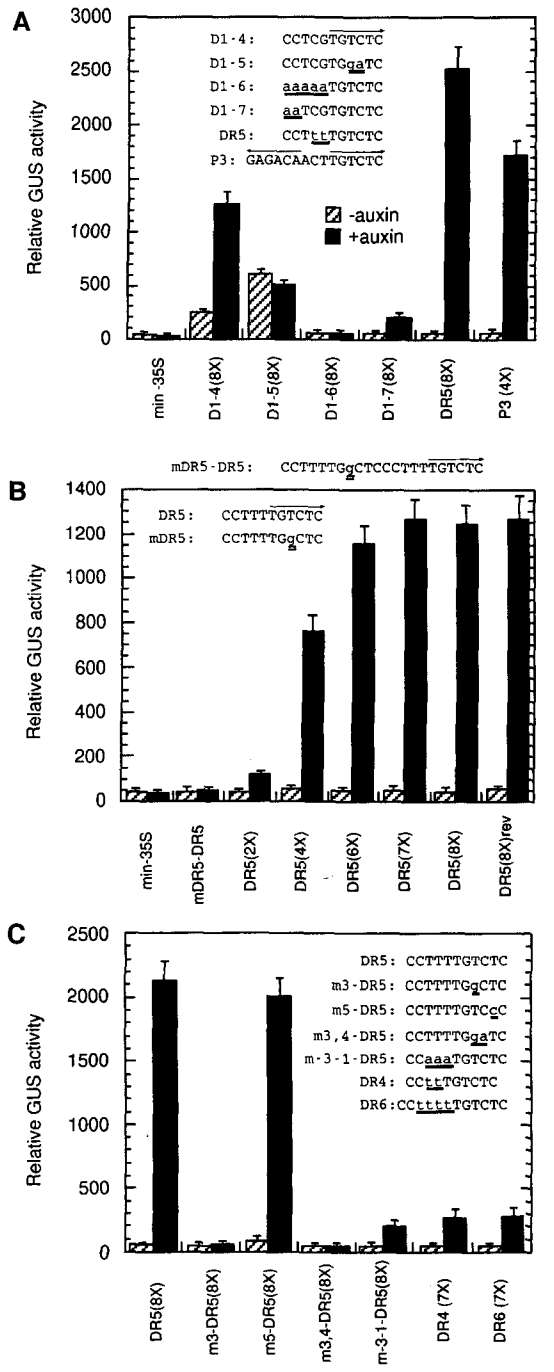

Figure 1. Quantitative Fluorometric GUS Assays for Auxin Inducibility of Synthetic AuxRE–*GUS* Reporter Genes.

(A) Nucleotide substitutions in the natural D1-4 AuxRE that create the synthetic DR5 AuxRE. Oligonucleotides used to make tandem four (4×) and eight (8×) copy constructs are shown at the top. Mutant nucleotides are indicated in lowercase letters. P3(4×) is a construct containing four palindromic repeats of the TGTCTC element and has been described previously (Ulmasov et al., 1997). min-35S is a −46 CaMV 35S promoter–*GUS* reporter gene. Transient assays in carrot protoplasts were performed as described by Liu et al. (1994) and Ulmasov et al. (1995). Auxin treatments were 25 μM 1-NAA for 24 hr.

(B) Effect of DR5 copy number on AuxRE activity. Oligonucleotides used to make the constructs are shown at the top. The number of DR5 tandem copies is indicated by 2X through 8X (i.e., two tandem copies to eight tandem copies). mDR5-DR5 is the same as the DR5(2×) but with a single mutant nucleotide in one of the two TGTCTC elements. DR5(8×)rev is the DR5(8×) construct in inverse orientation.

(C) Nucleotide substitutions in the DR5 AuxRE. Oligonucleotides used to make seven- or eight-tandem copy constructs are shown at the top.

Underlining indicates nucleotide differences from the D1-4 construct in **(A)** and from the DR5 construct in **(B)** and **(C)**. In **(A)** and **(B)**, arrows indicate forward or backward orientations of the TGTCTC element. Standard errors are indicated above bar graphs.

and showed increased constitutive expression with no auxin responsiveness. A 2-bp substitution (D1-7[8×]) and a 5-bp substitution (D1-6[8×]) outside of the TGTCTC element resulted in partial and complete loss of activity and auxin inducibility, respectively. In both of these mutant constructs, deoxyadenosines were substituted for the natural residues. In striking contrast, a 3-bp mutation with thymidine substitutions adjacent to the TGTCTC element (DR5[8×]) resulted in a substantial decrease in basal activity (i.e., equivalent to the minimal 35S construct) and a twofold increase in auxin-responsive activity. The synthetic DR5(8×) AuxRE was induced 25- to 50-fold by 1-NAA compared with fivefold induction with the natural D1-4(8×) AuxRE. The DR5(8×) construct, which contained tandem direct repeats of TGTCTC, also showed greater auxin inducibility than did the previously characterized P3(4×) construct (Ulmasov et al., 1997), which contained four palindromic repeats (i.e., eight copies of the TGTCTC element).

To determine the effect of copy number on DR5 AuxRE activity, single-copy and multiple tandem-copy constructs were assayed in carrot protoplasts. The single-copy construct (mDR5-DR5) contained two copies of DR5, with a single base pair substitution at position 3 in one of the copies. Because this substitution rendered the DR5 AuxRE inactive (see Figure 1A), the mDR5-DR5 construct in effect represents a one-copy construct that is directly comparable to a two-copy DR5(2×) construct.

Figure 1B shows that the single-copy construct had activity approximately equal to the minimal 35S construct and was not auxin responsive, whereas a two-copy construct was induced approximately threefold by auxin. Increasing the copy number to four in the DR5(4×) construct resulted in a several-fold increase in auxin inducibility. Further increases in auxin responsiveness were observed with six-copy DR5(6×) and seven-copy DR5(7×) constructs, but an eight-copy DR5(8×) construct was no more inducible than the seven-copy construct. An eight-copy DR5(8×)rev construct con-

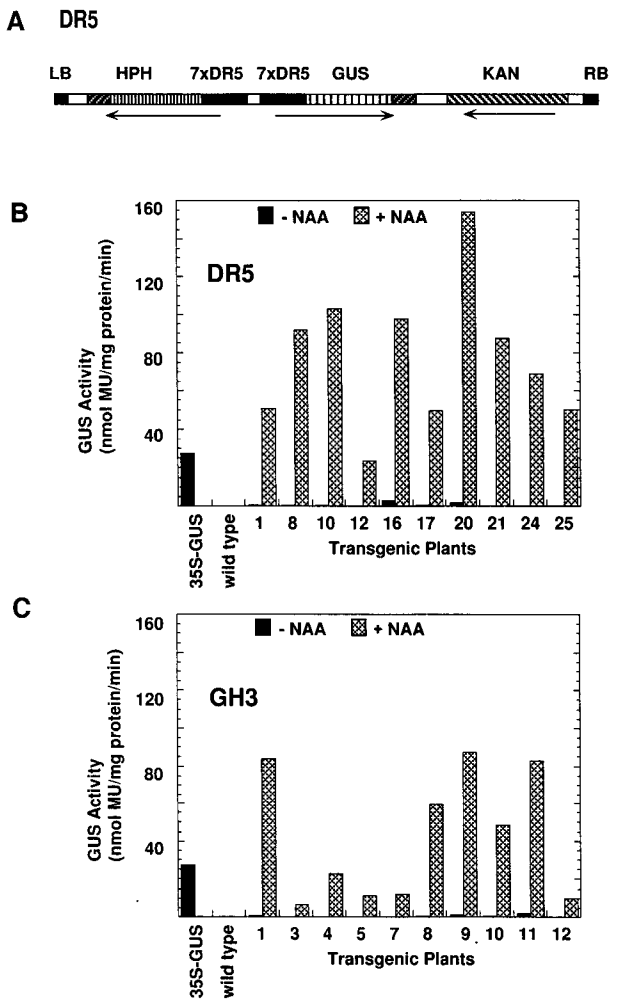

Figure 2. Quantitative Fluorometric Assays for GUS Activity in Arabidopsis Seedlings Transformed with DR5(7×)–*GUS* or *GH3–GUS*.

(A) Diagram of the T-DNA region of the binary vector containing the DR5(7×)–*GUS* construct. Seven copies of DR5 were ligated upstream of a minimal 35S promoter, which was used to drive both the *GUS* and the hygromycin phosphotransferase (*HPH*) genes. Both chimeric genes were terminated by the nopaline synthase 3′ untranslated region. The vector also contained a kanamycin resistance gene (*KAN*) for selection of transgenic plants. The arrows below the diagram indicate the direction of transcription for each gene. LB, left T-DNA border; RB, right T-DNA border.

(B) GUS-specific activities in plants transformed with the DR5(7×)–*GUS* construct. Six-day-old T_2 seedlings from individual transgenic lines were treated for 24 hr with H_2O (black bars) or 50 μM 1-NAA (hatched bars) and then assayed for GUS activity. Control assays were performed using nontransformed, wild-type Arabidopsis seedlings or seedlings transformed with a construct containing the 35S promoter ligated to the *GUS* gene (35S-GUS). Control plants were treated with H_2O only. Bar graphs represent the average of duplicate assays. MU, 4-methylumbelliferone.

(C) GUS-specific activities in plants transformed with a *GH3–GUS* construct. Arabidopsis plants were transformed with a construct similar to that shown in **(A)** but with the full-length (511 bp) soybean *GH3* promoter in place of DR5(7×). Plants were treated as described in **(B)**. Bar graphs represent the average of duplicate assays.

taining the DR5 AuxRE in inverse orientation was as active and auxin responsive as the DR5(8×) construct. These results with DR5 indicate that although copy number is important for auxin responsiveness, there is no effect of AuxRE orientation on promoter activity and auxin responsiveness, and there is little effect of copy number on basal (minus auxin) promoter activity.

To further characterize the DR5 AuxRE, site-directed mutations were made within and outside of the TGTCTC portion of DR5, and eight tandem-copy constructs were tested in carrot protoplasts. Nucleotide positions within 5′-TGTCTC-3′ are referred to as positions 1 to 6. A 1-bp mutation at position 3 (m3-DR5) and a 2-bp substitution at positions 3 and 4 (m3,4-DR5) resulted in total loss of activity and auxin responsiveness (Figure 1C). A single base pair substitution at position 5 (m5-DR5) had little effect on auxin responsiveness. These results are consistent with previous results showing that TGTCTC is required for auxin responsiveness but that positions 1 to 4 are more critical than are positions 5 and 6 (Ulmasov et al., 1997). Substitution of three deoxyadenosine residues for three thymidine residues adjacent to TGTCTC resulted in almost complete loss of auxin inducibility, indicating that sequence flanking the TGTCTC element is important for AuxRE activity. Addition (DR6[7×] construct) or subtraction (DR4[7×] construct) of a single thymidine residue adjacent to TGTCTC resulted in a several-fold loss in auxin responsiveness compared with a DR5(7×) construct (see Figure 1B) or DR5(8×) construct, suggesting that spacing between TGTCTC elements and/or nucleotide composition upstream of TGTCTC elements is important for auxin inducibility in the DR5 construct.

The DR5(7×) AuxRE was also tested for auxin responsiveness in stably transformed Arabidopsis plants. The DR5(7×) AuxRE was placed upstream of a minimal 35S promoter–*GUS* reporter gene (Figure 2A), and the reporter gene was transferred into Arabidopsis by using Agrobacterium vacuum infiltration (Bechtold et al., 1993). Ten 1-week-old independent transformants (T_2 generation) were assayed for GUS activity. Quantitative GUS assays showed that all transformants tested were induced >50-fold after treatment with 50 μM 1-NAA, with an average of ~100-fold induction (Figure 2B). Auxin-induced activity was generally greater with the DR5–*GUS* reporter gene than that observed with the auxin-responsive 511-bp *GH3* promoter–*GUS* reporter gene (Figure 2C).

Figure 3 shows histochemical staining for a DR5 transformant that was or was not treated with 1-NAA for 24 hr and subsequently stained for GUS activity over a period of 8 or 16 hr. The shorter staining period revealed that GUS activity was greatest in roots, but the longer staining period showed

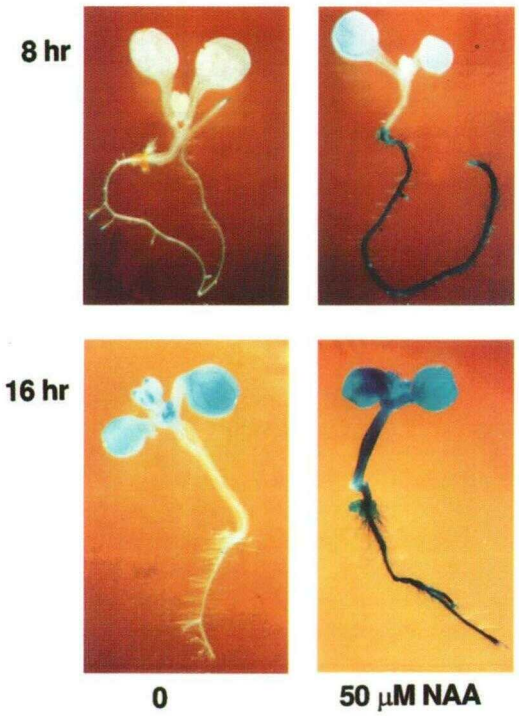

Figure 3. Histochemical Staining for GUS Activity in Arabidopsis Seedlings Transformed with DR5(7×)–*GUS*.

Data are shown for a single representative transgenic Arabidopsis line (plant line 8 in Figure 2B). Seedlings were treated for 24 hr with 50 μM 1-NAA (right) or H_2O (left) and then stained for GUS activity for ~8 hr (top) or 16 hr (bottom).

that GUS activity was detectable throughout the seedlings. This staining pattern was similar to that observed with the 592-bp *GH3* promoter–*GUS* reporter gene after auxin treatment of transgenic tobacco (Hagen et al., 1991; Liu et al., 1994) and Arabidopsis seedlings (data not shown). These results showed that the synthetic DR5 AuxRE functioned both in transient assays and in stably transformed plants. In addition, the DR5 AuxRE functioned in most if not all cell types when seedlings were exposed to exogenous auxin. The DR5(7×)–*GUS* reporter gene provides a useful reporter gene for studies on auxin-responsive gene transcription in Arabidopsis.

Gel Mobility Shift Assays with ARF1 and Aux/IAA Proteins

We have previously shown that auxin response transcription factors ARF1 and IAA24 (or ARF5) bind with specificity in vitro to a synthetic palindromic P3 AuxRE and a natural D0 AuxRE from the soybean *GH3* promoter (Ulmasov et al., 1997). Gel mobility shift assays shown in Figure 4 demonstrate that ARF1 also binds to the DR5 AuxRE in vitro. The binding efficiency of ARF1 to DR5 was less than that observed with the P3 AuxRE, suggesting that the preferred binding site for ARFs in these in vitro assays was an everted repeat as opposed to a direct repeat of the TGTCTC element. Although the DNA binding domain in ARF1 has been mapped to its N terminus and lies outside of domains III and IV and the amphipathic βαα motif that are conserved in Aux/IAA proteins (Ulmasov et al., 1997), these domains or other domains in the Aux/IAA proteins might nevertheless promote binding of Aux/IAA proteins to TGTCTC AuxREs.

To test this possibility, in vitro–translated Aux/IAA proteins were tested in gel mobility shift assays with a labeled DR5 probe. Figure 4 shows that under the same conditions that ARF1 bound DR5, Aux/IAA proteins, including soybean Aux22, soybean GH1, and Arabidopsis IAA13, failed to bind the DR5 probe. These results indicate that ARF1 and other ARF proteins are DNA binding proteins that recognize TGTCTC AuxRE target sites but that Aux/IAA proteins do not recognize and bind to these same AuxREs, at least under the in vitro conditions used in our experiments.

Interaction of ARF1 and Aux/IAA Proteins in a Yeast Two-Hybrid System

In an earlier study, we used the yeast two-hybrid system to clone an ARF1 binding protein (ARF2) with the C-terminal domain of ARF1 as bait (Ulmasov et al., 1997). This result suggested that domains III and/or IV in ARF proteins might

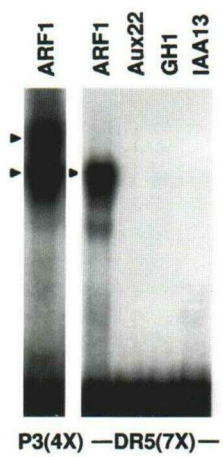

Figure 4. Gel Mobility Shift Assays with a DR5 Probe and ARF1 or Aux/IAA Proteins.

Either a ^{32}P-labeled P3(4×) or DR5(7×) probe was used with in vitro–translated ARF1, Aux22, GH1, or IAA13 proteins. The free probe is visible at the bottom of the gel, and complexes with ARF1 protein are shifted upward (arrowheads).

Table 1. Quantitative Measurements of GAL1–LacZ Transactivation by Hybrid GAL4 Proteins

Plasmids		
GAL4 DNA Binding Domain Fusion[a]	GAL4 Activation Domain Fusion[a]	β-Galactosidase Activity[b]
pAS1–Aux22	pACTII–ARF1-C534	68
pAS1–GH1	pACTII–ARF1-C534	110
pAS1–IAA12	pACTII–ARF1-C534	85
pAS1–IAA13	pACTII–ARF1-C534	116
pAS1–PsIAA4/5	pACTII–ARF1-C534	51
pAS1–SAUR6B	pACTII–ARF1-C534	<1
pAS1–GH3	pACTII–ARF1-C534	<1
pAS1–IAA12	pACTII–B36a[c]	<1
pAS1–GH1	pACTII–B36a	<1

[a] The GAL4 DNA binding domain in pAS1 and activation domain in pACTII are as described by Durfee et al. (1993).

[b] Relative β-galactosidase activity was measured in triplicate by using a chemiluminescence assay. Enzymatic activities represent the number (in thousands) of photons counted on a luminometer (model 3010; Analytical Scientific Instruments, Alameda, CA).

[c] B36a is the third largest subunit of RNA polymerase II from Arabidopsis.

be protein–protein interaction domains. Because these domains are also conserved in Aux/IAA proteins, we tested whether the C-terminal domain in ARF1 could interact with Aux/IAA proteins in a yeast two-hybrid system. Table 1 shows that Aux/IAA proteins do interact with the C-terminal domain in ARF1 but not with a control RNA polymerase II B36a protein in vivo. SAUR and GH3 proteins, which represent two other types of auxin-responsive proteins, do not interact with the ARF1 domain. These results suggest that ARF proteins, which target TGTCTC AuxREs through an N-terminal domain, may interact with ARF and Aux/IAA proteins through a conserved C-terminal domain. These results also suggest that Aux/IAA proteins might interact with each other because they all contain the conserved C-terminal domains III and IV.

Cotransfection Assays with Aux/IAA Effector Plasmids and AuxRE Promoter–*GUS* Reporter Genes

Because Aux/IAA proteins are capable of interacting with ARF proteins, which bind TGTCTC AuxREs, we tested whether overexpression of Aux/IAA proteins in carrot cells had any affect on transcription with DR5 reporter genes and other TGTCTC AuxRE reporter genes. An effector plasmid encoding the soybean Aux/IAA protein Aux22 was cotransfected into carrot cells with different reporter genes. Reporter genes with TGTCTC AuxRE promoters included the D1-4 natural AuxRE (Ulmasov et al., 1995), a synthetic composite AuxRE consisting of a chicken cRel DNA binding site fused upstream of TGTCTC (cRT; see Methods), and synthetic P3 (Ulmasov et al., 1997) and DR5 AuxREs. Expression of Aux22 from the effector plasmid resulted in repression of expression with each of these reporter genes (Figure 5A). Repression was approximately three- to fourfold in each case compared with reporter genes that were tested in the absence of an effector plasmid. Repression was specific for auxin-responsive transcription with TGTCTC AuxREs. The constitutive expression displayed by the D1-3 construct, which was derived from the D1 AuxRE, and by the D4-4 construct, which was derived from the D4 AuxRE (Ulmasov et al., 1995), was not affected by the effector plasmid. Likewise, reporter constructs driven by the soybean *GH2/4* octopine synthase (*ocs*) element (Ulmasov et al., 1994) and the Arabidopsis RNA polymerase II 19.5 subunit promoter (Ulmasov and Guilfoyle, 1992) showed no response to overexpression of Aux/IAA proteins. Although the *ocs* element represents an AuxRE and responds to auxin in carrot cells, it is not a TGTCTC AuxRE.

To test whether repression of auxin-responsive gene expression from TGTCTC AuxREs was a general characteristic of Aux/IAA proteins, an Aux22 effector plasmid was compared with pea Ps-IAA4/5, pea Ps-IAA6, and soybean GH1 (GenBank accession number AF016633) effector plasmids. Overexpression of each Aux/IAA protein resulted in a similar three- to fourfold repression of DR5 AuxRE–*GUS* reporter gene expression (Figure 5B). Repression was only observed when Aux/IAA proteins were produced because an antisense construct (DR5 + Aux22 antisense) and an out-of-frame construct (DR5 + GH1 out-of-frame) failed to cause repression of reporter gene expression. The antisense and out-of-frame constructs ruled out indirect repression that might result from DNA binding sites in effector plasmids titrating out some factor required for reporter gene expression. In contrast to effector plasmids that encoded Aux/IAA proteins, cotransfection with effector plasmids encoding several other auxin-responsive proteins, including soybean GH2/4, GH3, and SAUR 6B, had no effect on DR5 reporter gene activity. Overexpression of the Arabidopsis AXR1 protein (Leyser et al., 1993), which was used as another control, also had no effect on DR5 reporter gene expression.

Thus, repression of TGTCTC AuxRE activity appears to be a general characteristic of Aux/IAA proteins. These experiments should be interpreted with some caution, however, because repression with Aux/IAA proteins was observed when these proteins were overexpressed. Nevertheless, the repression is specific for reporter genes that contain a functional TGTCTC AuxRE and is consistent with in vitro results on interactions between ARFs and Aux/IAA proteins.

DISCUSSION

The synthetic DR5 element represents an exceptionally active AuxRE compared with natural composite AuxREs

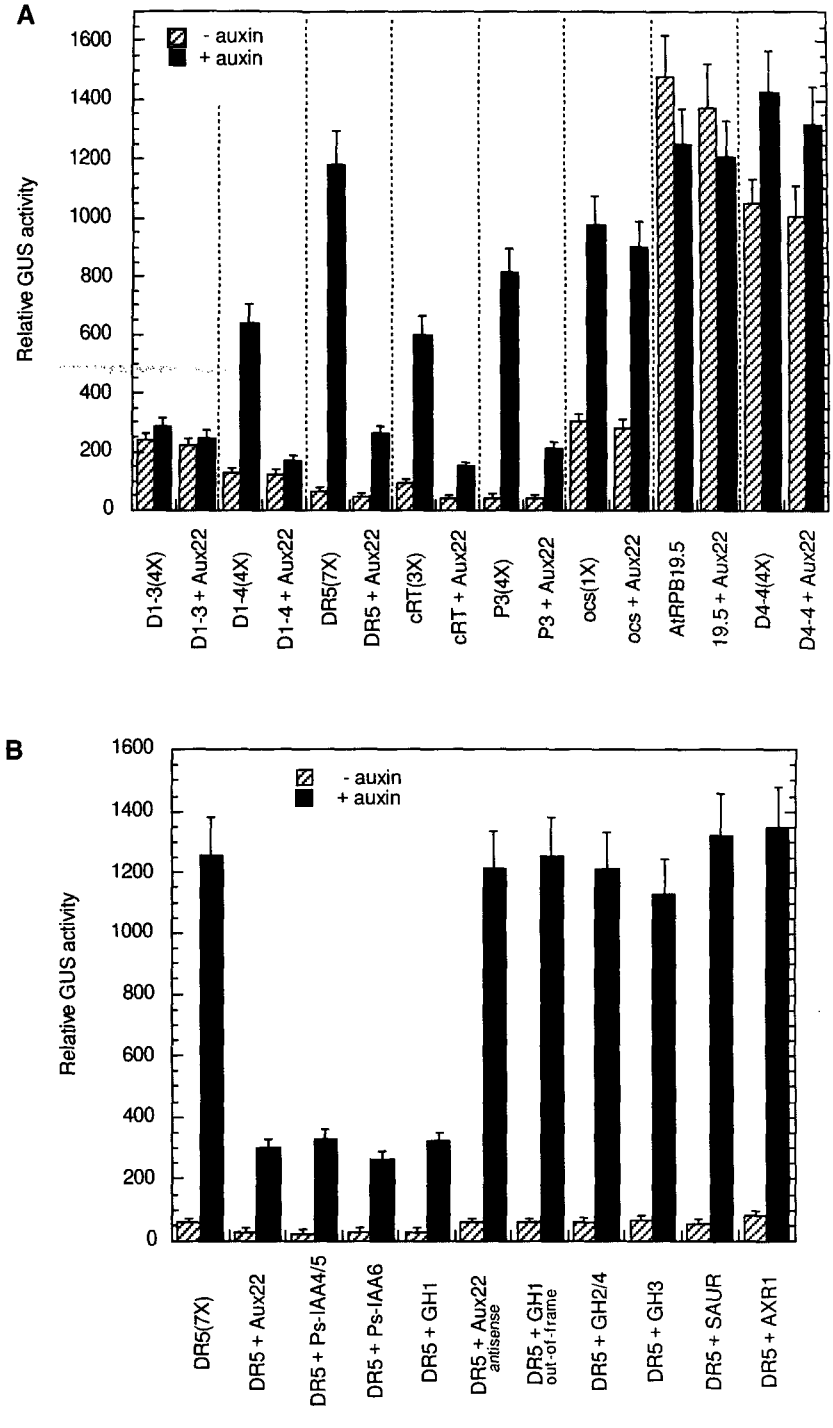

Figure 5. Specific Repression of TGTCTC AuxRE–Reporter Gene Expression by Overexpression of Aux/IAA Proteins.

(A) Overexpression of Aux22 represses only TGTCTC-containing promoters. Four TGTCTC AuxRE reporter genes were tested, and these included the natural D1-4 AuxRE (Ulmasov et al., 1995) and the synthetic DR5, P3 (Ulmasov et al., 1997), and cRT AuxREs. The cRT AuxRE is a composite AuxRE containing a chicken cRel DNA binding site fused to a TGTCTC element (see Methods). Two reporter genes consisted of mutant D1 (D1-3) and D4 (D4-4) AuxREs that were not auxin responsive but displayed constitutive activity (Ulmasov et al., 1995). Other reporter constructs contained promoters from the auxin-responsive soybean *GH2/4* 20-bp *ocs* element (Ulmasov et al., 1994) and the Arabidopsis RNA polymerase II 19.5-kD subunit (494 bp; Ulmasov and Guilfoyle, 1992). The effector plasmid consisted of a 35S promoter with a TMV 5′ leader fused to the Aux22 ORF (see Methods).

containing the TGTCTC element (Liu et al., 1994; Ulmasov et al., 1995) and a synthetic P3 TGTCTC AuxRE (Ulmasov et al., 1997). Based on results presented here, the DR5 element must be present in at least two tandem copies to function as an AuxRE in carrot protoplasts, and multimerization of this element up to seven copies further enhances the response to applied auxin. In a previous study, we synthesized and characterized another synthetic AuxRE, P3, which was composed of palindromic repeats of the TGTCTC element (Ulmasov et al., 1997). Results with both P3 and DR5 indicate that TGTCTC elements can function as AuxREs when multimerized and properly spaced as palindromic repeats or direct repeats in either orientation. It is also apparent from results with DR5 that the sequence adjacent to direct repeats of TGTCTC is important for auxin responsiveness.

Whether these sequence requirements result from the formation of novel coupling elements that overlap with the TGTCTC element or are an intrinsic property of direct repeats in simple AuxREs (i.e., AuxREs that are not dependent on a coupling element) is not clear. The palindromic P3(4×) construct described by Ulmasov et al. (1997) appears to function in the absence of any coupling element, and it is possible that the direct repeats in DR5 can also function without a coupling element. The organization of TGTCTC AuxREs as simple (i.e., direct or inverted repeats of TGTCTC not dependent on a coupling element) or composite elements may be analogous to steroid hormone response elements in animals that can be organized as simple (i.e., palindromic or direct repeats of a 6-bp steroid hormone response element) or composite elements (Yamamoto et al., 1992).

Synthetic AuxREs such as P3 and DR5 are much more active than natural AuxREs, and this provides several advantages for studying the molecular mechanisms of auxin-responsive gene expression. For example, the P3 AuxRE proved advantageous as bait in a yeast one-hybrid system to isolate an ARF1 transcription factor that bound with specificity to TGTCTC AuxREs (Ulmasov et al., 1997). The P3 AuxRE has also proved beneficial for characterizing the in vitro binding requirements of ARF proteins and for functionally defining DNA sequence requirements for AuxRE activity in vivo (Ulmasov et al., 1997). In this study, the DR5 element has been useful for defining the sequence requirements in TGTCTC AuxREs that function as direct repeats and for demonstrating that Aux/IAA proteins specifically repress auxin-responsive gene expression that is dependent on TGTCTC AuxREs. The DR5(7×)–*GUS* reporter gene construct in transgenic Arabidopsis provides a useful marker gene for studying auxin-responsive gene transcription in both wild-type plants and mutants. Because DR5 functions through a single type of TGTCTC AuxRE, it provides a more specific and better characterized marker than natural auxin-responsive promoters, which may contain multiple AuxREs and other *cis* elements that regulate gene transcription. The DR5 element has also been used in combination with a *GUS* reporter gene and hygromycin selectable marker gene (see construct in Figure 2A) to identify auxin-responsive mutants in Arabidopsis seedlings that contain these transgenes (J. Murfett, unpublished results).

Aux/IAA proteins have been hypothesized to be a family of transcription factors, possibly repressor proteins, involved in auxin-responsive gene expression (reviewed in Abel and Theologis, 1996). These proteins are thought to be DNA binding proteins with a DNA binding domain related to the amphipathic $\beta\alpha\alpha$ motif in Arc and MetJ repressor proteins (Abel et al., 1994). Although our results do not support Aux/IAA proteins functioning as DNA binding proteins on TGTCTC AuxREs, results with DR5, P3, and the natural D1-4 AuxRE indicated that overexpression of Aux/IAA proteins in carrot cells causes repression of TGTCTC AuxRE activity. At the same time, overexpression of these proteins had little to no effect on expression of other reporter genes, including those that contained *ocs*-type AuxREs. We propose that this repression by Aux/IAA proteins results from direct interaction between Aux/IAA proteins and ARFs and that these interactions are brought about by C-terminal domains containing conserved boxes III and IV found in both Aux/IAA and ARF proteins (Ulmasov et al., 1997). It may be that repression observed by overexpression of Aux/IAA proteins results from interactions between Aux/IAA and ARF proteins that either prevent ARFs from binding to TGTCTC AuxREs or prevent ARFs, when bound to TGTCTC AuxREs, from interacting with cofactors that are required for AuxRE activity. It is also possible, however, that overexpressed Aux/IAA proteins interact with and titrate out some other cofactors besides ARFs that are required for AuxRE activity. Whatever the case may be, our results show that several different Aux/IAA proteins act as repressors of TGTCTC AuxRE–GUS reporter gene expression and that this repression probably occurs without Aux/IAA proteins binding directly to TGTCTC elements.

Figure 5. (continued).

(B) Repression of DR5 AuxRE requires expression of functional Aux/IAA proteins. Seven tandem copies of the DR5 AuxRE were fused to a −46 CaMV 35S promoter–*GUS* reporter gene, and the reporter gene was tested for auxin inducibility in the presence or absence of effector plasmids that encoded Aux/IAA proteins (i.e., Aux22, Ps-IAA4/5, Ps-IAA6, and GH1), other auxin-inducible proteins (i.e., GH2/4, GH3, and SAUR 6B), or AXR1 (see Methods). The DR5 + Aux22 antisense and DR5 + GH1 out-of-frame constructs represent antisense and out-of-frame constructs that fail to produce functional Aux22 and GH1 proteins, respectively. Transient assays with carrot protoplasts were performed as described by Liu et al. (1994) and Ulmasov et al. (1995). Auxin treatments were 25 μM 1-NAA for 24 hr. Standard errors are indicated above bar graphs.

METHODS

Reporter Gene Constructs Used for Carrot Protoplast Transient Assays

All reporter constructs were created by using standard cloning procedures (Sambrook et al., 1989) and checked by automated DNA sequencing. Multiple copies of oligonucleotides (Gibco BRL) were cloned into SrfI or StuI sites upstream of the −46 cauliflower mosaic virus (CaMV) 35S promoter–β-glucuronidase (*GUS*) reporter gene (S2-46GUS vector; T. Ulmasov, unpublished results). DNAs for transfections were purified by the CsCl–ethidium bromide banding procedure (Sambrook et al., 1989). In the chicken cRel promoter–*GUS* reporter gene (cRT), three copies of the sequence GGGGAATTCCCCTGTCTC were fused upstream of a minimal −46 CaMV 35S promoter–*GUS* reporter gene (Ulmasov et al., 1995). This promoter consisted of the cRel DNA binding site, GGGGAATTCCCC, and the AuxRE TGTCTC. The cRT reporter construct was auxin responsive when the cRel DNA binding site was fused to TGTCTC; however, a mcRT construct containing mutations in the TGTCTC element showed constitutive expression and no auxin responsiveness (T. Ulmasov, unpublished results). This suggested that some transcription factor(s) in carrot protoplasts must recognize the cRel DNA binding site.

Effector Gene Constructs Used for Carrot Protoplast Transient Assays

The Aux22 (JCW2; Ainley et al., 1988) open reading frame (ORF) was isolated by reverse transcription–polymerase chain reaction (RT-PCR) from first-strand cDNA made with mRNA isolated from auxin-treated soybean hypocotyls. Ps-IAA4/5 and Ps-IAA6 ORFs (Ballas et al., 1993; Oeller et al., 1993) were isolated by RT-PCR from first-strand cDNA from auxin-treated pea epicotyls. IAA12 and IAA13 (Abel et al., 1995) were made by using RT-PCR with total RNA from Arabidopsis seedlings. Another Aux/IAA cDNA, GH1, an auxin-responsive GH2/4 glutathione *S*-transferase cDNA (Ulmasov et al., 1995), an auxin-responsive GH3 cDNA (Hagen et al., 1991), and an auxin-responsive SAUR 6B cDNA (McClure et al., 1989) were cloned from auxin-treated soybean hypocotyls (Hagen et al., 1984; McClure and Guilfoyle, 1987), and these cDNAs were used in effector plasmid construction. An AXR1 ORF, which encodes an ubiquitin-activating enzyme-like protein (Leyser et al., 1993), was cloned by using RT-PCR from total RNA isolated from Arabidopsis seedlings. All ORFs were cloned into the 35S–T7–tobacco mosaic virus (TMV)–3′ nopaline synthase vector (i.e., CaMV 35S with a duplicated enhancer 35S promoter combined with the T7 phage promoter followed by the TMV Ω translational enhancer and the 3′ nopaline synthase untranslated region), which was derived from the pAGUS1 vector described by Skuzeski et al. (1990). The integrity of all ORFs was checked by in vitro translation of T7 RNA polymerase-generated TMV Ω ORF transcripts in a ^{35}S-supplemented rabbit reticulocyte in vitro translation system (Promega) followed by SDS-PAGE and autoradiography.

Transfections

Transfection of carrot protoplasts and GUS and luciferase assays were performed as described previously (Liu et al., 1994; Ulmasov et al., 1995). In cotransfection assays, equal amounts of reporter and effector constructs were used. In cases in which no effector construct was added, a CaMV 35S promoter–chloramphenicol acetyltransferase reporter construct was used to equalize the amount of DNA transfected into carrot protoplasts.

Gel Mobility Shift Assays

For gel mobility shift assays, in vitro–translated ARF1 and Aux/IAA proteins (1 μL) were incubated in 20 μL of binding reaction with 2 ng of ^{32}P-labeled DR5(7×) probe. The binding buffer contained 12 mM Hepes, pH 7.9, 50 mM KCl, and 1 mM DTT. After 30 min of incubation at room temperature, binding reactions were loaded on a 4% polyacrylamide gel (0.25 × TBE; Ausubel et al., 1996) and subjected to electrophoresis at 10 V/cm for 2 to 3 hr at 4°C and autoradiography.

Reporter Gene Constructs Used for *Arabidopsis thaliana* Transformation

The DR5–*GUS* construct contained seven copies of DR5 cloned upstream of a −46 CaMV 35S promoter with a TMV 5′ leader (S2-46GUS vector, which was derived from pAGUS1; Skuzeski et al., 1990; T. Ulmasov, unpublished results). The *GH3–GUS* construct, containing 511 bp of the soybean *GH3* promoter, was described previously (Liu et al., 1994). Both constructs contained the *GUS* ORF and the 3′ nopaline synthase terminator. Each construct was cloned into the binary Ti vector pBIN19 (Bevan, 1984). The Ti vectors used in this study also contained the same promoters linked to a gene encoding hygromycin resistance (data for these genes will be presented elsewhere). The Ti vectors were transferred to *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986) for plant transformation.

Plant Transformations and GUS Assays

Arabidopsis ecotype Columbia plants were transformed by vacuum infiltration (Bechtold et al., 1993). Second generation (T_2) seedlings were germinated on a medium containing half-strength Murashige and Skoog (1962) salts, 1% sucrose, and 0.6% agar and grown under continuous light for 6 to 7 days. Intact seedlings were transferred to either H_2O or H_2O containing 50 μM 1-naphthaleneacetic acid (1-NAA) and incubated with gentle shaking for 24 hr. For fluorometric assays, total soluble proteins were extracted from 20 to 30 seedlings in each batch. Fluorometric and histochemical GUS assays were performed as described by Jefferson (1987) and Hagen et al. (1991). After histochemical staining, seedlings were cleared in 70% ethanol.

Yeast Two-Hybrid System

A cDNA encoding the C terminus of ARF1 (amino acids 533 to 655), which contained domains III and IV conserved in Aux/IAA proteins, and Arabidopsis RNA polymerase subunit B36a were fused to the yeast GAL4 DNA binding domain pAS1 vector (Durfee et al., 1993; Ulmasov et al., 1997), and these were used as bait to test for interactions with Aux/IAA and control proteins. Aux/IAA Aux22, GH1, Ps-IAA4/5, IAA12, and IAA13 cDNAs as well as SAUR 6B and GH3 cDNAs were cloned into the pACT2 vector, which contained the GAL4 activation (Durfee et al., 1993). Two-hybrid interaction assays were performed in yeast, as described by Ulmasov et al. (1997), according to the protocols recommended by Ausubel et al. (1996).

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